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Genetic polymorphism, mapping, and characterization of isocitrate dehydrogenase in *Anopheles quadrimaculatus*

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ABSTRACT: The isocitrate dehydrogenase-2 (*Idh-2*) locus of *Anopheles quadrimaculatus* was analyzed genetically and the enzyme was characterized physiochemically. Three-point testcrosses involving chromosome 3 markers showed that in female hybrids the gene sequence and the map distances are: nonstripe (*st*)—6.8—*Idh-2*—43.5—short antenna (*Sa*). Reduced recombination frequencies were observed in male hybrids (*st*—3.4—*Idh-2*—25.5—*Sa*). *Idh-2* activity gradually increases during development and reaches a peak intensity in adults. Maximum enzyme activity of *Idh-2* was obtained at p*H* 7.5. One-minute heat treatment at 50°C caused about 50 percent reduction of IDH-2. Ethylene diamine tetraacetic acid (EDTA 5mM) and p-chloromercuribenzoate (pCMB 10^{-5}M) caused complete loss of activity of IDH-2, but pretreatment of the enzyme in situ with mercaptoethanol protected the activity of allozymes from inhibition by pCMB treatment.

IN THE MOSQUITO, *Anopheles quadrimaculatus* (Say) is a species complex composed of three recognized sibling species. At the present time, these species are designated as A, B, and C. Their separate identities have been established by hybridization crosses, chromosome analysis (Kaiser et al., unpub. data), and isozyme characterization (Lanzaro et al. and Narang et al., unpub. data). Due to the presence of pesticide resistance in natural populations, this species is being evaluated for the application of biological control, including genetic control methods. Genetic control methods are species specific[3] and the possibility of reproductively isolated and genetically differentiated sibling species must be considered in the initial theoretical evaluation of proposed genetic control schemes. Extensive cytogenetic studies on other anophelines, particularly *Anopheles gambiae* and *Anopheles maculipennis* showed that both taxa are species complexes[1,2,8,10]. It is therefore essential that a pest species be identified accurately through genetic studies[8] on chromosomal polymorphism, hybridization, and electrophoretic analysis of population genetic structure. Diagnostic allozymes of two isocitrate dehydrogenase (IDH) loci can be used to identify two sibling species, A and B. This paper reports the isozyme polymorphism in three natural populations and the developmental profile, properties of *Idh-1* and *Idh-2* electromorphs, and linkage relationship of isocitrate dehydrogenase-2 (*Idh-2*) in a laboratory colony of species A. The karyotype of *Anopheles quadrimaculatus* consists of two pairs of autosomes and one pair of heteromorphic sex chromosomes and the male is heterogametic.

Materials and Methods

Genetic mapping and enzyme polymorphism: The linkage of *Idh-2* to the stripe (st^+) mutant (LG III) was determined by a two-point testcross and was previously established (Lanzaro et al. unpub. data). We used an additional mutant, short antenna (*Sa*) in three-point testcrosses to determine the gene order of *Idh-2*, *st*, and *Sa* on chromosome 3. Two laboratory stocks of *A. quadrimaculatus* were used: 1) *Sa* st^+ $Idh\text{-}2^{132}$; heterozygous for the dominant mutant, short antenna (*Sa*)[6] (lethal when homozygous), and homozygous for the naturally occurring trait, stripe (st^+)[5] and for $Idh\text{-}2^{132}$; 2) Sa^+ *st*

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Idh-2^{100}. The crosses shown in Table II were used to determine the gene order on chromosome 3. IDH polymorphism was studied in four populations: three natural populations from 1) Gainesville, Florida; 2) Lake Charles, Louisiana; 3) Stuttgart, Arkansas; and a laboratory population.

Zymograms and characterization: Starch-gel electrophoresis in a tris-citrate buffer (CA-8)[9] system at p*H* 8.45 and a zymogram technique was used for analyzing isocitrate dehydrogenase (IDH). Ten individuals of three developmental stages (viz. 4th instar larvae, young and old male and female pupae, and young and old adults of the Q2 strain homozygous for *Idh-1*^{100} and *Idh-2*^{100}) were homogenized in 60 μl of grinding buffer (CA-8 gel buffer containing bromophenol blue) and centrifuged for 2 minutes at 12 000 rpm. The volume of each supernatant was adjusted to 80 μl with grinding buffer. Ten microliters of each sample was electrophoresed. Vertical polyacrylamide gel electrophoresis in CA-8 buffer system was run at 250 volts and 130 mA for 3 hours for characterization studies.

Physiochemical characterization of diagnostic allozymes of two *Idh* loci were undertaken to characterize these loci. Thermostability of isozymes was tested by sealing the gel in a plastic bag and incubating it at various temperatures (40°C–60°C) in a water bath for 1 to 15 minutes followed by quick cooling of the gel and staining for IDH. The p*H* range of enzyme activity was determined by incubating gel slices in stain solution with p*H* range of 6.4 (phosphate buffer) to 10.0 (tris-HCl buffer for 7.5 to 10.0). The effect of various inhibitors such as urea (2M), ethylene diamine tetraacetic acid (EDTA 5mM), p-chloromercuribenzoate (pCMB 10^{-5}M) was studied by incubating the gel in buffer containing various concentrations of respective inhibitor for 30 minutes at 4°C before incubation in the staining mixture, which also contained respective inhibitor. In order to determine the presence of sulfhydryl groups in the active site of the IDH enzyme, the gel was pretreated with 1mM of 2-mercaptoethanol for 30 minutes at 4°C prior to treatment with the inhibitor, pCMB, as described above. LKB Ultroscan XL laser densitometer was used to measure the effect of treatment on the relative intensity of IDH bands.

Results

Electrophoretic patterns and developmental changes

Two zones of IDH activity appear on zymograms of the developmental stages examined (Figures 1 and 2). The inheritance studies indicated that electromorphs at each zone are controlled by two independent loci, designated *Idh-1* and *Idh-2*. Both loci are polymorphic (Figure 1 and Table I) and the IDH bands of our standard marker stock, homozygous for *Idh-1*^{100}, and *Idh-2*^{100}, were used as standards (*Rf* = 100) to identify the allozymes at each locus as follows: *Idh-1* - *Rf* = *84*, *86*, *100*, and *112*; *Idh-2* - *Rf* = *100*, *132*, *162*, and *197*. The natural populations differ in allelic frequencies at both *Idh-1* and *Idh-2* loci (Table I). The Gainesville and Lake Charles populations are polymorphic at the

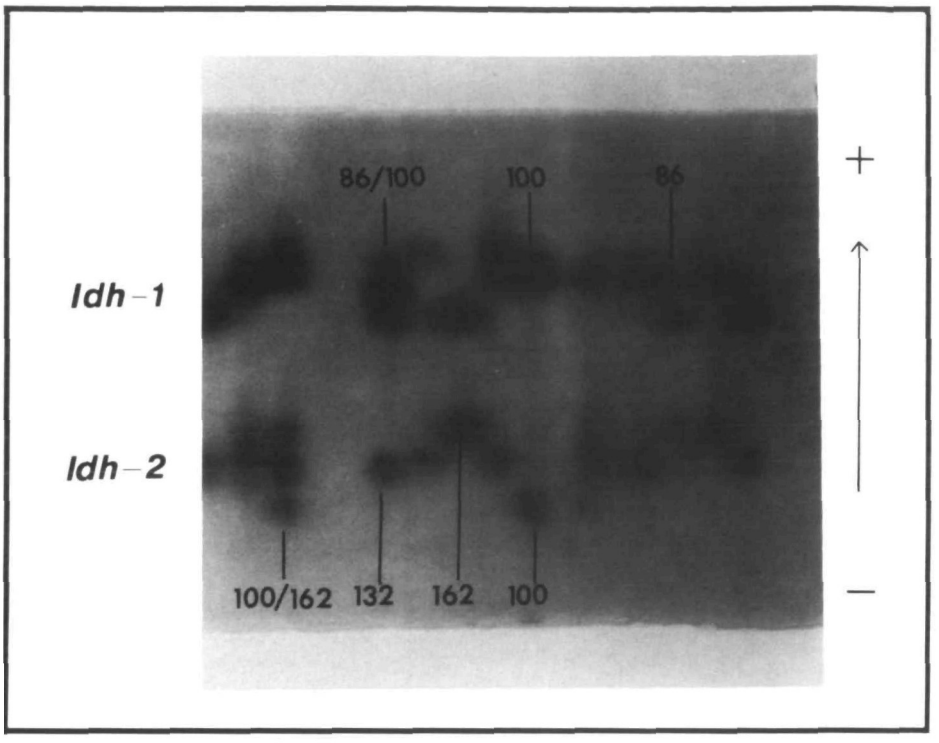

FIGURE 1 IDH zymogram showing electromorphs of two loci. Two alleles at *Idh-1* (*86*, *100*,) and three alleles (*100*, *132*, *162*,) at *Idh-2* locus.

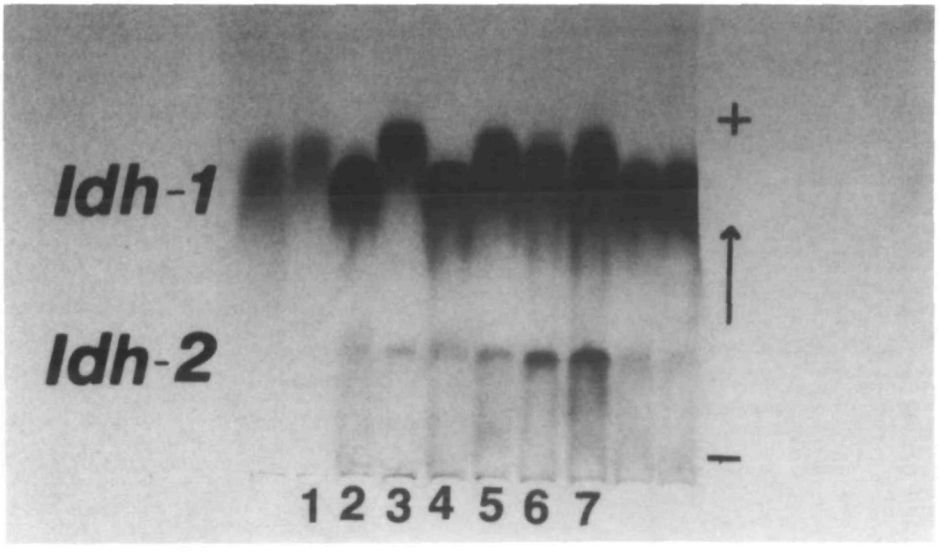

FIGURE 2 IDH zymogram showing developmental changes in the activity of *Idh-1* and *Idh-2* genes. Stages of mosquitoes: 1—fourth-instar larva; 2—young male pupa; 3—young female pupa; 4—old male pupa; 5—old female pupa; 6—young male adult; 7—young female adult.

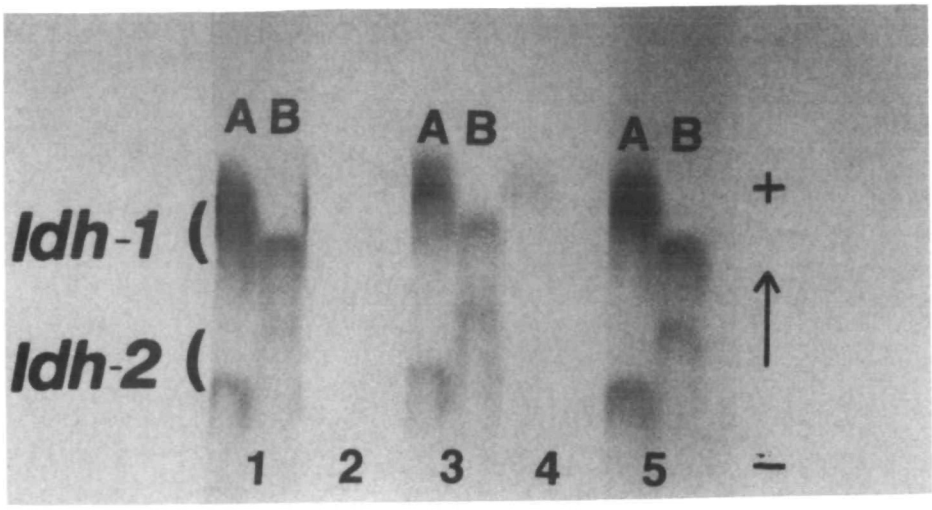

FIGURE 3 IDH zymogram showing effect of inhibitors on the activity of two loci. *A* and *B* designate sibling species A and B of *A. quadrimaculatus*, respectively. Inhibitors used: 1—control; 2—5mM ethylene diamine tetraacetic acid; 3—2M-Urea; 4—10^{-5}M p-chloromercuribenzoate; 5—pretreatment with 2-mercaptoethanol before treating with inhibitor p-chloromercuribenzoate.

Idh-1 locus, whereas the Stuttgart population (N = 27) showed only one allele, *Idh-1*^{100}. At the *Idh-2* locus, allele *100* is most common in Stuttgart (freq. = 0.58), but this allele occurs at low frequencies in Gainesville and Lake Charles, where *Idh-2*^{132} is most common. On the other hand, *Idh-2*^{162} is absent in Stuttgart, but present in Gainesville and Lake Charles. Chi-square analysis showed that all of the populations of species A were in Hardy-Weinberg equilibrium (Table I).

Table I. Allele frequencies and genetic variability at *Idh-1* and *Idh-2* loci in 4 populations of *A. quadrimaculatus*

Locus	Populations* KBG	ORL	LC	RB
Idh-1				
(N)	128	26	54	27
84	0.03	0.04	0.02	0.0
86	0.14	0.0	0.11	0.0
100	0.81	0.85	0.85	1.0
112	0.02	0.11	0.02	0.0
Idh-2				
(N)	104	23	25	26
100	0.06	0.48	0.02	0.58
132	0.55	0.50	0.78	0.42
162	0.31	0.02	0.20	0.0
197	0.08	0.0	0.0	0.0
Idh-1				
O (E)†	0.25 (0.32)	0.23 (0.27)	0.22 (0.26)	0.0 (0.0)
Idh-2				
O (E)	0.54 (0.59)	0.48 (0.52)	0.44 (0.36)	0.46 (0.49)

* KBG = Kanapaha Garden, Gainesville, Florida; ORL = Orlando Laboratory Colony; LC = Lake Charles, Louisiana, RB = Red Barn, Stuttgart, Arkansas

† O = Observed heterozygosity; E = Expected heterozygosity based on Hardy-Weinberg equilibrum

Figure 2 shows the developmental profile of IDH allozymes. IDH-1 activity is lower in larvae, but seemingly equal in the other developmental stages. The activity of IDH-2 gradually increases during development and reaches a peak intensity in the adults. More enzyme activity is observed in females than in males. Two-week-old adults have slightly (20 percent) more enzyme activity than 1-day-old adults. The relative intensities of the IDH-2 bands of various developmental stages, based on the densitometric analysis, were reproducible in different repetitions. Since the adults possess the highest IDH-2 activity (Figure 2), all the characterization studies were done on the adult enzyme.

Genetic mapping

In three-point testcrosses with hybrid females (Table II) the gene sequence and the map distances were: *st*—6.8—*Idh-2*—43.5—*Sa*. However, crosses with hybrid males yielded lower estimates: *st*—3.4—*Idh-2*—25.5—*Sa*.

Properties

Maximum enzyme activity of IDH-2 was observed in all developmental stages when the gel was stained at p*H* 7.5. The activity was reduced to about 50 percent, 70 percent, and 40 percent of maximum at p*H* 6.4, 9.0, and 10.0, respectively.

One-minute heat treatment of the gels at 50°C caused about 50 percent reduction of the IDH-2 enzyme activity and no activity was detected after 5 minutes at 50°C. On the other hand, IDH-1 showed no noticeable loss of activity even after 15 minutes at 50°C. No apparent effect was observed at 40°C whereas at 60°C isozymes at both loci were completely inhibited.

Figure 3 shows that pCMB (10^{-5}M) and EDTA (5 mM) caused complete loss of activity of IDH-1 and IDH-2. However, mercaptoethanol protected the allozymes from being inactivated by pCMB treatment. Urea (2M) did not inactivate the allozymes.

Discussion

The present study showed clear developmental differences in IDH-2 activity with maximum activity in the adults. The difference in the IDH-2 band intensity between males and females (Figure 2) may be due to a difference in weight between the sexes. Young female adults were on the average 1.6 times heavier than young males. Similar observations on IDH activity were noted for *Drosophila melanogaster*[4].

By combining the data in this report with other works[6] on the linkage map of the right arm of chromosome 3(3R) in *A. quadrimaculatus*, the current map of 3R is: centromere—*Sa*—43.5—*Idh-2*—6.8—*st*—9.4—yellow(*y*)—?—malathion resistance *mal*^{R}. The distance between *y* and *mal*^{R} is not known precisely, because the *mal*^{R} gene is inside a paracentric inversion. *Idh-1* was assigned to a position on the left arm of chro-

Table II. Results of three-point testcrosses

Cross*	Parental	Recombinants I	II	double	Genetic map distance
1	305	23	236	17	*st* 6.8 *Idh-2* 43.5 *Sa*
2	407	16	142	3	*st* 3.4 *Idh-2* 25.5 *Sa*
Total	712	39	378	20	*st* 5.1 *Idh-2* 34.6 *Sa*

* 1. F_1 (*st*$^+$ *Idh-2*^{132} *Sa* × *st Idh-2*^{100} *Sa*$^+$) × *st Idh-2*^{100} *Sa*$^+$; 4 families
2. *st Idh-2*^{100} *Sa*$^+$ × F_1(*st*$^+$ *Idh-2*^{132} *Sa* × *st Idh-2*^{100} *Sa*$^+$); 3 families

mosome 2 (Lanzaro, pers. comm.). The difference in recombination frequency recorded for males and females is similar to that observed in *A. albimanus*. Crossing over in males was 40–60 percent of that observed in females in crosses involving the markers on chromosome 2 of *A. albimanus*[7].

Using preliminary characterization criteria such as thermostability, p*H* optimum, and effect of inhibitors, EDTA and pCMB, we did not detect any major differences between $Idh\text{-}2^{100}$ and $Idh\text{-}2^{132}$ allozymes. This does not rule out differences in their physiological roles. Further biochemical tests will be needed to determine the differences in metabolic function if any.

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